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Sigma factor SigC is required for heat acclimation of the cyanobacterium *Synechocystis* sp. strain PCC 6803

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Abstract The role of the primary-like sigma factor SigC was studied in *Synechocystis*. Under high temperature stress (48 °C) the ΔsigC inactivation strain showed a lower survival rate than the control strain. The ΔsigC strain grew poorly at 43 °C in liquid cultures under normal air. However, change to 3% CO_2 enhanced growth of ΔsigC at 43 °C. Differences in expression of many genes related to the carbon concentrating mechanisms between the control and the ΔsigC strain were recorded with a genome-wide DNA microarray. We suggest that low solubility of CO_2 at high temperature is one of the factors contributing to the poor thermotolerance of the ΔsigC strain.

Keywords: Sigma factor; RNA polymerase; Regulation of transcription; Heat; Cyanobacteria

1. Introduction

Temperature is a key environmental factor. The optimum growth temperature of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is 30–32 °C, but the temperature range permissive for growth stretches from about 15 °C to a maximum of 43 °C [1]. For short times, *Synechocystis* cells can even tolerate temperatures up to 50 °C [1]. Exposure to non-lethal high temperature actually improves the overall thermotolerance in *Synechocystis* [2]. An immediate response to high temperature is the transient production of heat shock proteins.

The mechanism of acclimation to high temperature is only partly understood in *Synechocystis*. The histidine kinase Hik34 is a component of a negative regulatory pathway of some heat shock genes [3] and the CIRCE/HrcA system is involved in the regulation of the *groESL1* and *groEL2* genes encoding heat shock proteins [4]. The primary-like σ factor, SigB, is rapidly upregulated at high temperatures [5,6] and mediates short-term heat shock responses [7] apparently by regulating genes encoding heat shock proteins [4,7]. The alternative σ factor gene, *sigH*, shows up-regulation after long heat treatments, but the inactivation of *sigH* did not influence the performance of the cells at 45 °C [8]. In this paper, we show evidence that SigC plays a vital role in the thermotolerance of *Synechocystis*.

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Abbreviations: Chl, chlorophyll; PPFD, photosynthetic photon flux density

2. Materials and methods

2.1. Construction of inactivation strains

The glucose-tolerant strain of *Synechocystis* [9] was used as the control strain. The genomic DNA was isolated as in [9] and the *sigC (sll0184)* gene was amplified by PCR, using the primers 5′-ATG ACT AAA CCA AGC AAC GA-3′ and 5′-AAT CTA GCA AAA TTT CCT GC-3′. The PCR product was cloned into the pCR–Blunt II-TOPO vector (Invitrogen). The pCR–Blunt II-TOPO-sigC was digested with *Spe*I and*Eco*RV and the *sigC* fragment was ligated into the *Xba*I and *Sma*I double digested pUC19. To construct pUC19–sigC–Kn, the *Bam*HI fragment of pUC4K (Amersham Biosciences), carrying the Kn resistance cassette, was ligated into *Bgl*II digested pUC19–sigC. To construct pUC19–sigC–Ω, the *Bam*HI fragment of pHP45Ω [10] conferring streptomycin (Str) and spectinomycin (Spc) resistances, was ligated into *Bgl*II digested pUC19–sigC. Transformation was done according to a standard method [9]. The complete replacement of the native gene with the inactivated gene was confirmed by PCR analysis.

2.2. Growth measurements and heat treatments

Synechocystis cells were grown in BG-11 medium supplemented with 20 mM Hepes–NaOH pH 7.5 at 32 °C under the continuous photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1}. One hundred millilitres cell suspensions were grown in 500 ml erlenmyers, the cultures shaken at 90 rpm in a growth chamber at ambient air conditions. Plates for the inactivation strains were supplemented with antibiotics as in [7], but all physiological measurements were done with cells grown without antibiotics. At the start of the heat experiment, cells were resuspended to BG-11 medium to OD_{730nm} of 0.14 (5×10^6 cells/ml), and the cells were grown at 43 °C in normal air or in 3% CO_2, as indicated, under the PPFD of 35 μmol m^{-2} s^1. The cultures were shaken at 90 rpm. Growth was monitored by measuring OD_{730nm}. Cell viability after the heat treatments was measured as in [7]. To follow the inactivation of photosynthetic activity at 48 °C, the cell suspensions were heat treated at 48 °C under a PPFD of 5 μmol m^{-2} s^{-1} for 0, 5, 15, 30 and 45 min (heat treatments were done in dim light to avoid photoinhibition during the treatment). After the treatment, the sample was placed into the cuvette of an oxygen electrode (Hansatech, King's Lynn, UK) and supplemented with 10 mM

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$NaHCO_3$. The temperature was allowed to cool to 32 °C for 2 min under dim light, and the photosynthetic activity was then measured at 32 °C under saturating light (500 μmol photons m^{-2} s^{-1}).

2.3. Northern blot and reverse transcription PCR analysis of RNA

Cells were grown under standard conditions for 2 days (final OD_{730nm} was 0.6–0.7), harvested by centrifugation, suspended in fresh BG-11 medium (10 μg chlorophyll (Chl) ml^{-1}), and treated at 43 °C in normal air under the PPFD of 35 μmol m^{-2} s^{-1} as indicated. Control samples were taken before the heat treatments. Total RNA isolation and Northern blotting were performed as described before [11]. The gene specific probes were amplified from *Synechocystis* DNA by PCR.

For reverse transcription PCR, isolated RNA was treated with 5 units of RQ1 RNase-free DNase (Promega) for 2 h at 37 °C. Then cDNA synthesis was performed with the Ready-To-Go You-Prime First-Strand Beads kit (Amersham Biosciences). The reverse primer for *sigA* was 5′-AAAAGACGACGACGGAAGGC-3′ and for *sigC* 5′-TTTACCGTTTTGAGGGTTT-3′. PCR was done using the same reverse primer as in cDNA synthesis; the forward primer for *sigA* was 5′-ATGACCCAGACGAAAGAGCC-3′ and for *sigC* 5′-ATGACTAAACCAAGCAACGA-3′.

2.4. DNA microarray analysis

The cells were grown under standard conditions in normal air for 2 days (OD_{730nm} was 0.6–0.7), harvested by centrifugation, and resuspended in fresh BG-11 medium (10 μg Chl ml^{-1}). Cells of the control and ΔsigC strain were treated at 43 °C under the PPFD of 35 μmol m^{-2} s^{-1} for 24 h. Total RNA was extracted from 15 ml culture samples, after which DNA microarray hybridization and data analysis were performed essentially as in [12].

3. Results

3.1. Inactivation of both sigB and sigC genes makes Synechocystis cells extremely vulnerable to high temperature stress

We constructed an inactivation strain ΔsigC by interrupting the *sigC* gene in the *Synechocystis* genome with a Kn cassette. Construction of the ΔsigB strain was described in detail earlier [7]. The double inactivation strain ΔsigBC was constructed by inactivating the *sigC* gene in the ΔsigB strain with an Ω fragment. Complete segregation of ΔsigC and ΔsigBC strains was verified by PCR (Supplementary Fig. S1).

In cyanobacteria, photosynthesis is one of the most heat-sensitive processes. We followed the loss of photosynthetic activity at a lethal temperature, 48 °C, in the ΔsigC and ΔsigBC strains by measuring the light saturated photosynthetic activity with an oxygen electrode. For comparison, the same measurements were done for the control and ΔsigB strains. The faster loss of photosynthetic activity in the inactivation strains than in the control strain was obvious throughout the experiment and finally after 45 min of treatment at 48 °C, the remaining photosynthetic activity was down to 21% of the starting value in the control strain, and to 10% in ΔsigB and ΔsigC strains and only to 2% in the ΔsigBC strain (Fig. 1A).

Because photosynthesis was more vulnerable to high temperature stress in the inactivation strains than in the control strain, we tested the ability of the cells to survive at 48 °C. In the control strain, 20% of the cells survived a 15-min treatment at 48 °C, whereas the survival rates were 2% for ΔsigB and 4% for ΔsigC, respectively (Fig. 1B). The lowest survival rate of less than 0.1%, was measured in the ΔsigBC strain.

Next, we applied a 1-h pre-treatment at 43 °C that was sufficient to make control cells 100% resistant against a 15-min treatment at 48 °C. The survival rates of strains ΔsigB, ΔsigC and ΔsigBC were 45%, 66% and 25%, respectively, indicating that the pre-treatment improved the thermotolerance of the inactivation strains but none of the inactivation strains was able to reach acquired thermal tolerance in full (Fig. 1B).

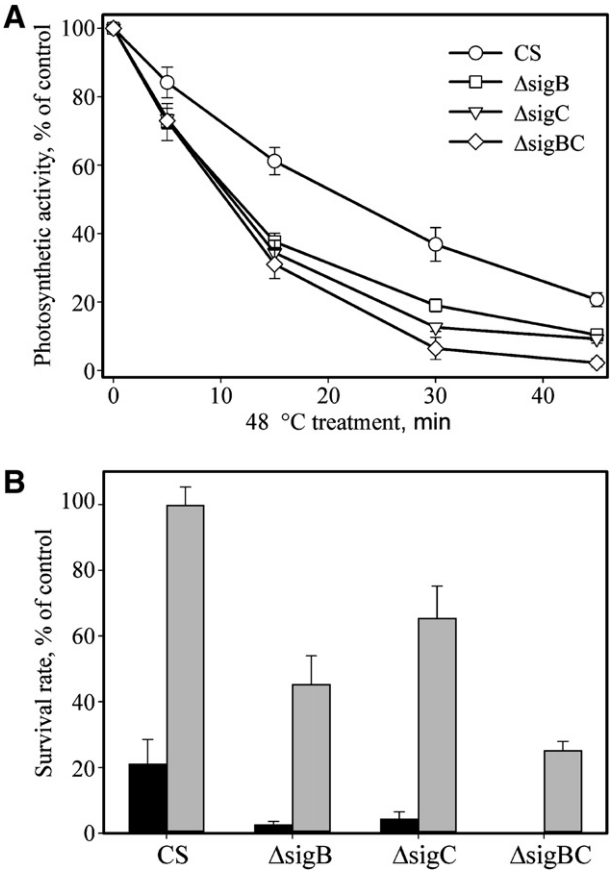

Fig. 1. Performance of the strains at 48 °C. (A) The loss of photosynthetic capacity at 48 °C. The cells were heat treated at 48 °C under a PPFD of 5 μmol m^{-2} s^{-1}. The photosynthetic activities before the heat treatments were 96.3 ± 5.3, 88.5 + 11.2, 83.2 + 6.8 and 88.3 + 9.9 μmol O_2 mg Chl^{-1} h^{-1} in the control (circles), ΔsigB (squares), ΔsigC (triangles) and ΔsigBC (diamonds) strains, respectively. Each experimental point is the result of at least three independent experiments and the error bars denote S.E. (B) The survival rate of the cells after a 15-min heat treatment at 48 °C under a PPFD of 5 μmol m^{-2} s^{-1} without a pre-treatment (black columns) and after a 1-h pre-treatment at 43 °C (grey columns). Each bar represents the mean of four independent experiments and the error bars denote the S.E.

3.2. Growth of the ΔsigC and ΔsigBC strains ceases at 43 °C

The growth of the inactivation strains was followed under sub-lethal high temperature conditions. The OD_{730nm} was set to 0.14 and the growth of the control, ΔsigB, ΔsigC and ΔsigBC strains was followed at 43 °C under the continuous PPFD of 35 μmol photons m^{-2} s^{-1}. The control and ΔsigB strains were able to grow at 43 °C although the growth rate became the slower, the longer the cells were kept at 43 °C (Fig. 2). Strains ΔsigC and ΔsigBC grew slowly only during the first day at 43 °C and thereafter the growth of these strains ceased completely.

We followed the changes in the amount of *sigC* transcripts at 43 °C by reverse transcription PCR. A control experiment (Fig. 3A) verified a linear relationship between the amount

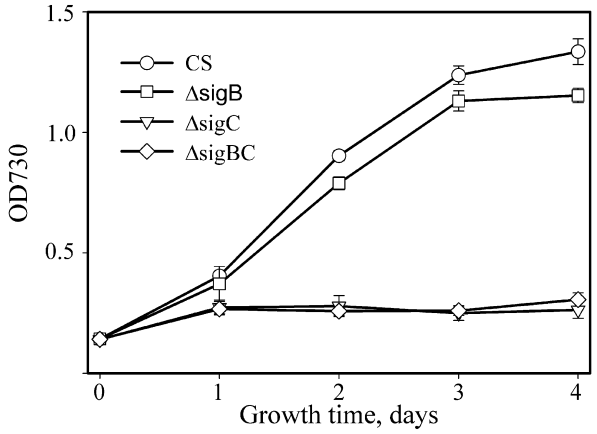

Fig. 2. Growth of strains at 43 °C. The OD_{730nm} of cell culture was set to 0.14, and the control (circles), ΔsigB (squares), ΔsigC (triangles) and ΔsigBC (diamonds) strains were grown at 43 °C under normal air conditions. Each growth curve represents the average of five independent experiments and the error bars denote the S.E.

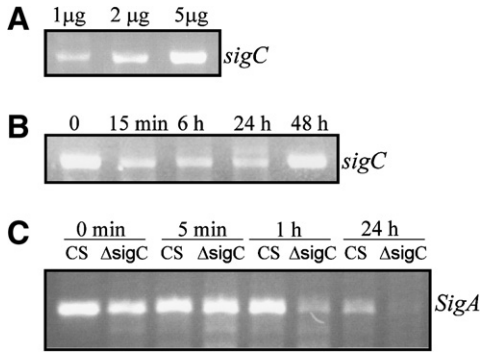

Fig. 3. The amounts of *sigC* and *sigA* transcripts at 43 °C. (A) Linear relationship between the amount of *sigC* transcripts in the sample and the 423 bp *sigC* PCR product in reverse transcription PCR; 1, 2 or 5 μg of total RNA was used for cDNA synthesis; 2 μl of PCR reaction was loaded in each well. (B) The amount of *sigC* transcripts after 0 min, 15 min, 6 h, 24 h and 48 h heat treatment at 43 °C in the control strain. One micrograms of total RNA was used for cDNA synthesis; 10 μl of PCR reaction was loaded in each well. (C) The amount of *sigA* transcripts after 0 min, 5 min, 1 h and 24 h heat treatments at 43 °C in the control and ΔsigC strains. One micrograms of total RNA was used for cDNA synthesis.

of *sigC* transcripts and the amount of the PCR product. The amount of *sigC* transcripts first decreased at 43 °C, but after 48 h a similar amount of *sigC* transcripts was detected as under standard growth conditions (Fig. 3B).

The primary σ factor SigA is mainly responsible for transcription of housekeeping genes during exponential cell growth. Because growth of the ΔsigC strain was retarded at 43 °C, we compared the expression of the *sigA* gene at 43 °C in the control and ΔsigC strains. The amount of *sigA* transcripts decreased in both strain at 43 °C, but this decrease occurred more rapidly and was more prominent in the ΔsigC strain that in the control strain (Fig. 3C).

3.3. Gene expression profiles at 43 °C

A genomewide DNA microarray was used to compare gene expression in the control and the ΔSigC strain. Genes with altered expression levels between ΔSigC and control strain after 24 h at 43 °C are listed in Table 1. The results for all 3264 genes are shown in Supplementary Table S1. The genes whose expression levels were higher in the ΔsigC strain than in the control strain include the operon *ndhF3-ndhD3-cupA* that has been suggested to be involved in low-CO_2-inducible CO_2 uptake [13]. The transcript levels of the sodium-dependent bicarbonate transporter gene *stbA* and its adjacent gene *stbB*, a periplasmic protein gene *slr1247*, a pilin polypeptide PilA1 gene and an operon including two flavoprotein genes (*sll0217-sll0219*) were higher in ΔsigC than in the control strain. Furthermore, five genes with unknown function were expressed more in ΔsigC strain than in the control strain. Only three genes had a notably lower expression level in the ΔsigC strain than in the control strain after 24 h of heat stress (Table 1).

According to our DNA microarray data, the expression levels of all heat shock genes were similar in the control and ΔsigC strains. To verify that the SigC factor does not directly regulate the synthesis of heat-specific genes, transcripts of the *groESL1* operon and *groEL2* and *hspA* genes were measured with Northern blot technique. Concomitant with the array results, differences between the control and ΔsigC strains proved minor only (Fig. 4).

Interestingly, 11 of the 15 genes (i.e. *sll0217–sll0219*, *slr1512–slr1513*, *sll1732–sll1734*, *sll1694*, *slr0006*) that were more upregulated in ΔsigC than in the control strain at 43 °C were among the 20–25 genes that are strongly and permanently upregulated when *Synechocystis* cells are transferred from 3% CO_2 to ambient CO_2 [14].

3.4. Availability of inorganic carbon and growth performance at high temperatures

Using the temperature dependency of Henry's law for CO_2, the equilibrium concentration CO_2 at 43 °C can be estimated to be 76% of the concentration at 32 °C. We studied the role of CO_2 in high temperature acclimation by growing the control and ΔsigC strain at 43 °C in air supplemented with 3% CO_2. Both the control and ΔsigC strain grew well for the first day at 43 °C in CO_2 enriched atmosphere (Fig. 5). The enhanced growth in 3% CO_2 (Fig. 5), compared to normal air (Fig. 2), was especially clear in the ΔsigC strain that hardly grew at all without added CO_2. During the second day, growth of both strains became slower, this retardation being more prominent in the ΔsigC strain than in the control strain. Growth of both strains completely ceased after the second day at 43 °C. These findings suggest that the inability of the ΔsigC strain to grow at high temperature under normal air level CO_2 is partly dependent on low availability of inorganic carbon at high temperature and partly by another, yet unknown, factor.

4. Discussion

The ΔsigC strain is temperature sensitive which became clear from a number of observations including hampered growth at 43 °C in normal air, relatively fast loss of photosynthetic capacity under heat stress, and a low survival rate after a short heat treatment at 48 °C. These results indicate a role of SigC factor in heat stress management. In the control strain, however, heat-treatment did not specifically induce expression of

Table 1
Differentially regulated genes in the ΔsigC and control strains measured after 24 h treatment at 43 °C

Gene	Gene function	(ΔsigC − CS)/CS	*P*-value
sll0217	Flavoprotein	1.62	1.25E−03
slr0364	Hypothetical protein	1.45	1.66E−04
slr1247	Phosphate-binding periplasmic protein precursor (PBP)	1.43	3.89E−08
sll1733	NADH dehydrogenase subunit 4 (involved in low CO_2-inducible, high affinity CO_2 uptake)	1.38	1.87E−05
slr1512	Sodium-dependent bicarbonate transporter	1.35	5.76E−03
slr1513	Periplasmic protein, function unknown	1.16	1.79E−02
slr0581	Unknown protein	1.16	2.32E−04
ssr1528	Hypothetical protein	1.13	3.02E−03
sll0219	Flavoprotein	1.13	3.57E−03
sll1734	Involved in low CO_2-inducible, high affinity CO_2 uptake	1.13	1.21E−03
sll0218	Hypothetical protein	1.12	1.26E−02
slr0006	Unknown protein	1.08	1.87E−02
sll1694	Pilin polypeptide PilA1	1.00	1.88E−07
slr1259	Hypothetical protein	0.99	6.62E−06
sll1732	NADH dehydrogenase subunit 5 (involved in low CO_2-inducible, high affinity CO_2 uptake)	0.97	1.09E−04
sll0108	Ammonium/methylammonium permease	−1.07	1.51E−07
ssl2501	Unknown protein	−1.05	1.16E−04
ssl2384	Unknown protein	−1.00	2.28E−02

The fold values were calculated by dividing the difference in the intensities of the ΔsigC and control strains and dividing the result by the intensity of the control strain. Given values are the means of three independent experiments using RNA isolated from separate cultures.

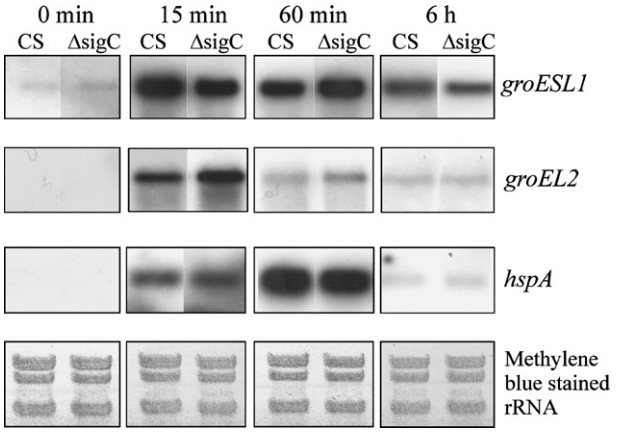

Fig. 4. Northern blots showing the heat-induced accumulation of *hspA*, *groESL1* and*groEL2* transcripts in the control and ΔsigC strains at 43 °C. Total RNAs were isolated before (0 min) and after 15 min, 60 min or 6 h treatments at 43 °C. Five micrograms of RNA was loaded in each well and the amounts of *hspA*, *groESL1* and*groEL2* transcripts were detected in the control (CS) and ΔsigC strain with gene specific DNA probes by Northern blot analysis. Northern blots shown are representatives of three independent heat treatments. Equal loading and even transfer were verified by methylene blue staining of the membrane.

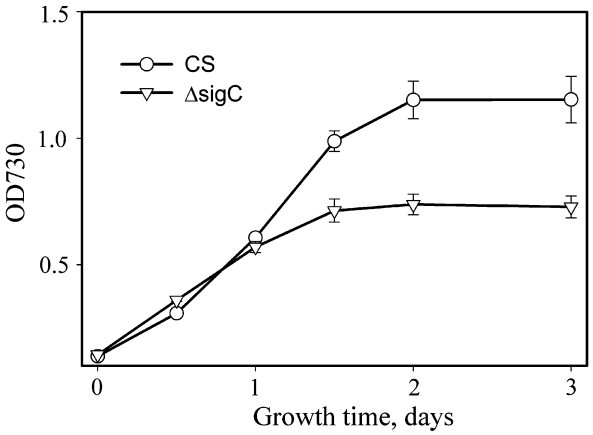

Fig. 5. Growth of control and ΔsigC strains at 43 °C under high CO_2. The OD_{730nm} of cell culture was set to 0.14, and the control (circles) and ΔsigC (triangles) strains were grown at 43 °C under high (3%) CO_2 conditions. Each growth curve represents the average of three independent experiments and the error bars denote the S.E.

the *sigC* gene (Fig. 3). DNA microarray data (supplement) and Northern blot experiments revealed that the heat shock genes were expressed similarly in ΔsigC and control strains. This suggests that the SigC factor is not required for normal up-regulation of the heat shock genes, as different from SigB factor of *Synechocystis* that is actually involved in regulation of heat shock genes [4,7]. The role of the SigC factor in acclimation of *Synechocystis* to high temperature thus appears different from the roles of σ^{32} in *Escherichia coli* [15] and σ^{B} in *Bacillus subtilis* [16] which recognize specific promoter sequences upstream of genes encoding heat shock proteins.

We suggest that at least three different phenomena affect the inability of the ΔsigC strain to grow at 43 °C. Firstly, reduced growth is related to the inability of the ΔsigC strain to produce normal amounts of *sigA* transcripts under heat stress. The relationship between the amount of the primary σ factor and growth performance has been illustrated in *E. coli* by studying mutants in which the expression of the *rpoD* gene encoding the σ^{70} factor was controlled using the promoter of the tryptophan operon [17]. In these experiments the underproduction of the primary σ^{70} factor in *E. coli* reduced the growth rate of the cells [17]. Secondly, the notion is that less CO_2 is dissolved in the growth medium at high temperatures than at normal growth temperature. The finding that many genes related to carbon concentrating mechanisms are differently regulated in the ΔsigC and control strain, suggests that the SigC factor might have a role in carbon balancing processes. The observed down-regulation of an ammonium/methylammonium permease gene in ΔsigC strain in DNA microarray analysis (Table 1) may be indicative of a disequilibrated C to N balance in

ΔsigC strain at high temperature. The role of SigC in the carbon regulation cascade is strengthened by the finding that the ΔSigC strain can grow as well as the control strain at 43 °C for one day if the air is supplemented with 3% CO_2. Thirdly, the observed differences in cell densities between the control and ΔSigC strains after prolonged heat treatments can be partially assigned to differences in stationary phase between these strains. It has been shown that the SigC factor in *Synechocystis* and its homolog SigE factor in *Synechococcus* sp. PCC 7002 contribute to gene expression and survival of the cells at the stationary phase [18,19].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.12.030.

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